Retinoic Acids Promote the Repair of the Dermal Damage and the Effacement of Wrinkles in the UVB-Irradiated Hairless Mouse

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Chronic irradiation of hairless mice with UVB leads to elastosis as evidenced by both histologic means and an increase in skin desmosine content. Treatment with topical all-trans- or 13-cis-retinoic acid causes dose-dependent increments in the area of the dermal "repair zone"; skin desmosine content increases during irradiation but does not change significantly after irradiation is discontinued and retinoic acid treatment commenced. During the course of the irradiation the animals develop permanent wrinkles on the exposed dorsal surface, which can be recorded in plastic impressions. The extent of wrinkling can be quantitated and it has been demonstrated that topically applied retinoic acids lead to the complete effacement of these surface features and that the process appears to be permanent. *J Invest Dermatol 91:175–180, 1988*

Chronic exposure of the skin to sunlight or ultraviolet radiation causes severe damage to the underlying connective tissue, typically manifested in elastosis and alterations in glycosaminoglycans and collagen [1–4]. It is generally accepted that such actinic damage is a major cause of the more rapid "aging" of exposed areas of the skin compared to sun-protected areas [5]. The UV-irradiated hairless mouse is now the species of choice in many laboratories for a variety of photobiologic investigations. The action spectrum and time-course for UV-induced erythema in this mouse are similar to the sunburn response in humans [6]. UV-induced connective tissue damage is also similar to that observed in man [1] and is described in detail by Kligman & Kligman [7]. Once considered to be irreversible, this damage was recently observed to undergo a substantial degree of spontaneous repair; upon cessation of the UV irradiation, a band of new dermal tissue was laid down in the immediate subepidermal region [1,8] compressing the old elastotic tissue. Thus the measure of repair is reflected in the width of this "zone of reconstruction" [9]. Of particular interest was the finding that topically applied all-trans-retinoic acid, in a dose-dependent manner, greatly augmented the rate of the repair process.

We have recently found that chronic exposure of these animals to UVB radiation, in amounts that cause dermal elastosis and damage, leads to the appearance of wrinkles. These take the form of regularly spaced furrows on the dorsal surface. Because these wrinkles do not disappear upon gentle stretching, they are distinguished from the array of regularly spaced lines seen on non-irradiated animals at sites where the excess skin normally lies in folds. The term "permanent wrinkle" has been applied to such deep wrinkles in sun-exposed human skin.

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Abbreviations:
UV: Ultraviolet
UVB: Ultraviolet B (290–320 nm)

We report here the effects of topically applied all-trans- and 13-cis-retinoic acids on the repair of UVB-induced dermal damage and effacement of wrinkles.

MATERIALS AND METHODS

Animal Treatment Schedules Hairless mice (female, HRS/J strain, Jackson Labs; 5–7 weeks old at the start of the experiments) were housed in yellow light and irradiated three times per week with a bank of eight Westinghouse Sunlamps (FS40) placed about 20 cm above the animals. The radiation dose was controlled by an International Light Model IL844A Phototherapy Exposure Control and a model SEE240 detector. The UVB dosing schedule was such that individual doses, seldom exceeding 0.06 J/cm^2, caused minimal erythema but no burning or scarring. There was significant elastosis, detected by histology after a total dose of about 3.5–4.0 J/cm^2 (accumulated over a period of 5–6 months); this was confirmed in measurements of elastin in whole skin by means of a radioimmunoassay for desmosine, an elastin-specific amino acid found in hydrolysates of elastin and considered to be a reliable index of total elastin content.

Radioimmunoassay of Desmosine Desmosine was determined by a modification of the procedure of King et al [10] using an antiserum kindly provided by Dr. Barry Starcher, University of Texas Health Center (Tyler, Texas). Briefly, a 4 mm punch of whole skin, free from subcutaneous fat, was heated at 105° for 48 h in a sealed tube containing 1 ml of constant boiling HCl (Pierce). After evaporation to dryness the residue was taken up in 1 ml water and stored at −70° until assay. The tracer was I^{125} labeled desmosine-Bolton Hunter Reagent (Elastin Products, Inc., Pacific, MO) that was prepared according to the manufacturer's instructions. Standard curves were set up covering a range of 0.02 to 5.0 ng desmosine and appropriate amounts of sample were added to a dilution of antiserum, which bound about 40% of the tracer. After incubation overnight at 4°, bound desmosine was separated by precipitation with 50% ammonium sulfate and quantitated by gamma counting. Calculations of desmosine content were done by conventional methods using a program written to interpolate values from the linearized standard curve. Results are quoted in ng desmosine/

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mm^2 skin. The sensitivity of the assay run with about 3000 dpm of freshly prepared tracer was of the order of 0.02 ng. Typically, with the described irradiation schedules, desmosine increased by about twofold after a dose of about 3.5 J/cm^2 of UVB. Whole skin (dermis plus epidermis) was used because it had been shown that epidermis contains no detectable desmosine.

Retinoid Treatment To effect repair of the dermal damage, the UVB irradiation was discontinued and the animals were divided into groups of approximately eight and treated three times per week with various concentrations of the retinoids dissolved in acetone. Stock solutions were made up freshly every week in subdued light at concentrations such that the dose was delivered in 100 μl volume and applied topically with a plastic pipette to an area of about 10 cm^2 on the back of the animal. All dosing was done under yellow light. A control group treated with acetone alone was included. Between applications, solutions were stored under argon at −70°. After 10 weeks of treatment the animals were killed and skin samples were taken and processed by standard methods.

Histology Two-centimeter strips of dorsal skin, taken laterally across the center of the irradiated (and treated) area, were fixed in 10% formalin, embedded in paraffin and sectioned at 6 microns. Hematoxylin/eosin was used for routine examination of the tissue; elastin fibers were stained with Luna's aldehyde fuchsin [11] and collagen by Van Gieson. A typical example of tissue from a treated animal is shown in Fig 1 (Luna stained). In this model, repair is defined by the appearance of a normalized dermis extending from the epidermis down to the layer of compressed elastin. The extent of repair is reflected by the width of this zone. In our studies, because the width of the zone varies considerably, we measured the area of the zone on a standard length of histologic section by an image analyzer (Image Technology Corporation). Twenty-four fields, each of which (at 100X magnification) represents a section of tissue 0.57 mm long, were examined and the area calculated. The results are reported as the average area per field in mm^2. Thus the average width of the repair zone can be obtained by dividing this average area by 0.57 mm. Areas of substantial repair are interspersed with areas with no discernible repair. This "focal" nature of the effect is reflected in large standard deviations and increments that sometimes do not reach statistical significance. All microscopic fields were included in the calculations of average area (or width). Data were analyzed by Student's t-test.

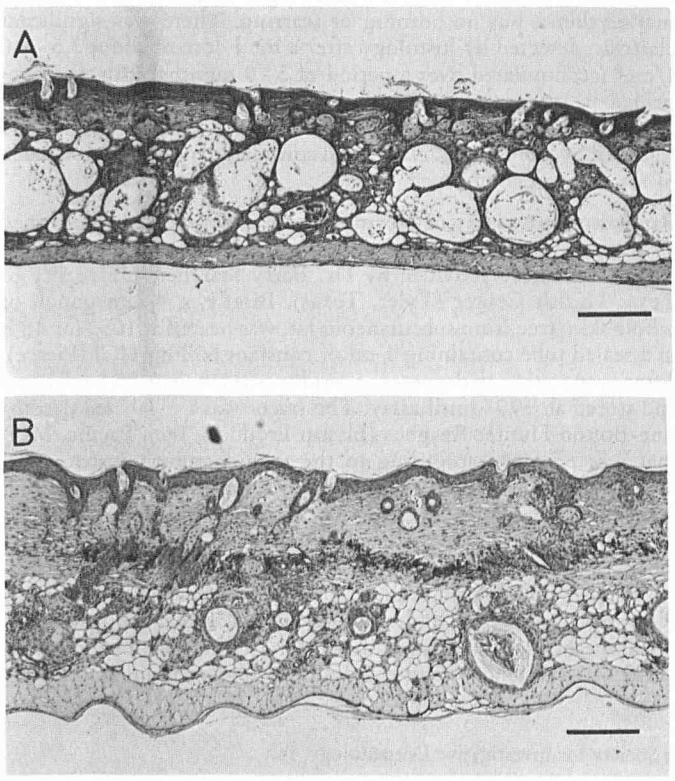

Figure 1. Histologic sections of tissue from UVB-irradiated animals treated with either vehicle *(A)* or 100 μg 13-cis-retinoic acid *(B)*. Luna stained: ×40. The *bar* represents 200 μm.

Wrinkle Measurements Skin replicas were taken of the lower dorsal area by means of SILFLO (Flexico Developments Ltd., England). The animals were anesthetized with Chloropent (Fort Dodge Laboratories, Fort Dodge, Iowa) (0.1 ml/animal, i.p.). An adhesive electrode ring (Novametrix, Wallingford, CT) with a 1-cm diameter hole was placed on the surface that had been irradiated and/or treated, and the SILFLO was applied according to the manufacturer's instructions. It was possible to place the adhesive ring on the same part of each animal's back by positioning it relative to the base of the tail. Wrinkles appear in these impressions as ridges and cast a shadow when illuminated with low-angle light. A characteristic of the wrinkling pattern is the occurrence of furrows in a regularly spaced array about 2-3 mm apart. The extent of wrinkling was assessed using this line pattern by assigning values (the Wrinkle Index), on a scale of 0 to 4, to the width of shadow, which is proportional to the depth of the wrinkle, with 0 representing the absence of wrinkling and 4 the greatest degree of wrinkling (Fig 2). Samples were read in a blind manner by two independent observers and the results were pooled. It should be emphasized that only the regularly spaced transverse wrinkle pattern was used for quantitation; other fine structure elements were not considered. In some instances impressions were taken from the same animal at separate times; the period between sampling was 2 weeks, which allowed time for the mild irritation caused by the procedure to subside. These surface replicas are relatively easy to obtain and provide a better source for analysis of topographical features than photographs of the intact animals. The spacing of the wrinkle pattern is such that 3 or 4 wrinkles are included in the area covered by the replica; thus the latter adequately represents the condition of the animal from which it was taken.

Procedures to analyze the skin impressions have been developed for an image analyzer (Image Technology Corporation). In this case the total area of shadow, of defined intensity, cast by the wrinkle is computed. This method yields essentially identical results as visual assessment.

RESULTS

Figure 1 illustrates the histologic appearance of skin from UVB-irradiated animals treated with either vehicle *(A)* or 100 μg of 13-cis-retinoic acid *(B)*. As can be seen there is negligible "repair" in the vehicle-treated tissue and the elastin is found over the entire dermis, particularly at the epidermal/dermal junction. In contrast, the "repair zone" in the treated tissue varies in width from about 100 to 220 μm. In this species, the non-irradiated epidermis is about 16 μm thick and increases to about 32 μm after UVB irradiation. Further increases are induced by the action of retinoids with values typically in the range of 35–50 μm, as seen in Fig 1*B*. In some instances the epidermis has projections into the dermis, usually associated with sebaceous glands, but their low frequency does not significantly affect the overall measurements of the repair zone width. Total skin thickness (dermis plus epidermis) is about 440 μm in non-irradiated animals, increasing to about 570 μm after UVB irradiation. Retinoid treatment causes further thickening to values in the range of 800–1000 μm. Thus, despite epidermal hyperproliferation, the major cause of the skin thickening is the dermal fibroplasia.

As shown in Table I, a total dose of UVB of 3.5 J/cm^2 caused an approximate doubling of the skin desmosine content. This degree of elastosis was consistent with the histologic findings, where the numbers of elastic fibers appeared to have increased by about twofold. When the irradiation was discontinued, no further changes in

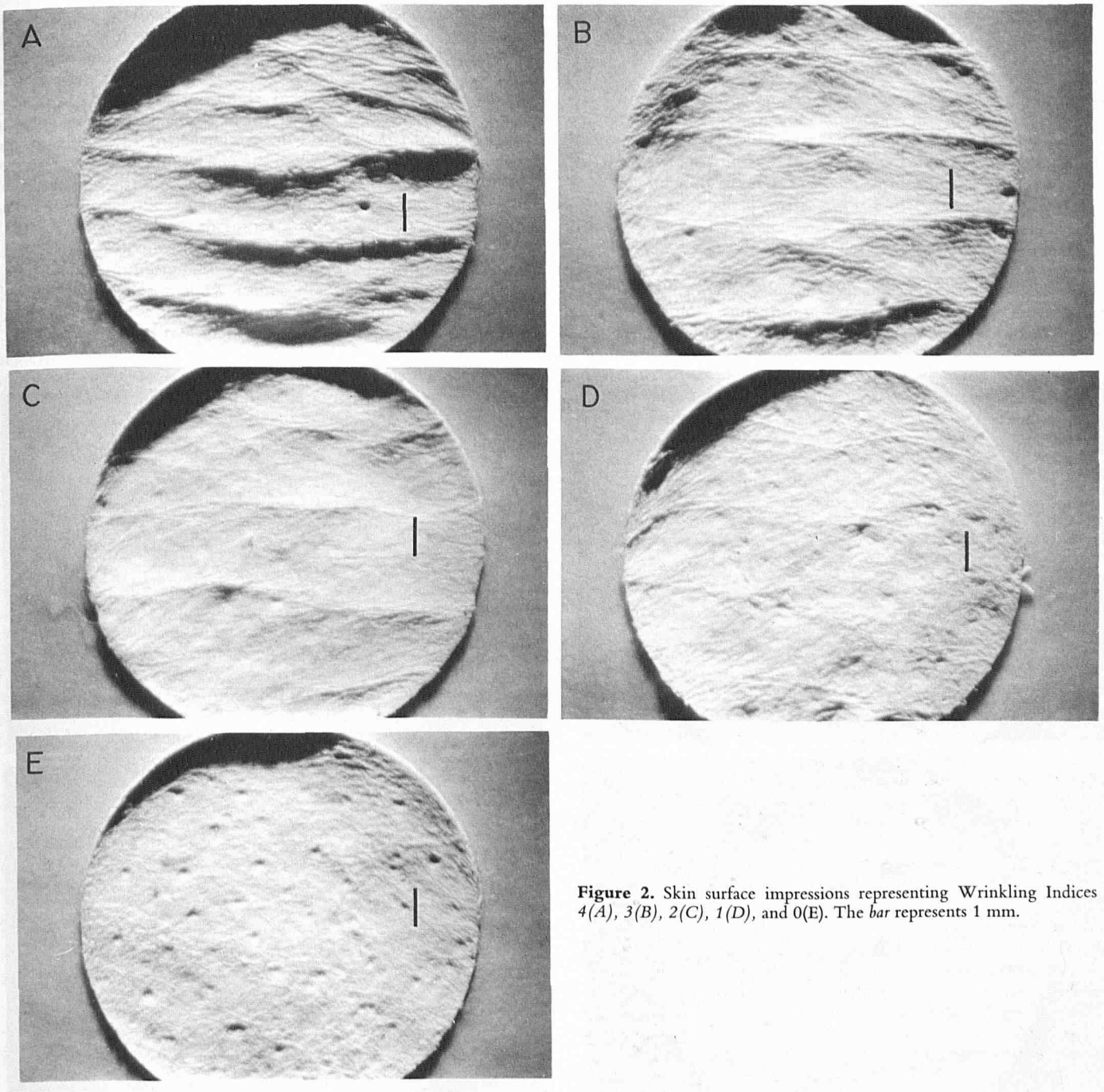

Figure 2. Skin surface impressions representing Wrinkling Indices of *4(A), 3(B), 2(C), 1(D),* and 0(E). The *bar* represents 1 mm.

desmosine were observed either in control or treated groups; this was also consistent with the histologic appearance.

All-trans- and 13-cis-retinoic acids were found to produce dose-related increments in the area of the dermal repair zone (Fig 3). 13-cis-Retinoic acid appears less potent but, at equivalent doses, less irritating. The maximum response may be higher for the 13-cis isomer by virtue of the higher dose that could be used. As seen from the figure the width of the repair zone increases from about 20 μm in controls to about 70 μm at the highest doses of retinoids. All-trans-retinoic acid is very toxic to the animals at doses higher than 25 μg (previous studies had shown that, in our hands, a dose of 50 μg was lethal after 4 weeks of treatment; in the same dosing schedule, up to 400 μg of 13-cis-retinoic acid was tolerated for up to 10 weeks).

Figure 4 shows the surface patterns of non-irradiated mouse skin, UVB-irradiated mouse skin, and skin from hairless mice that had been irradiated and then treated with either vehicle (control) or

Table I. The Effect of UVB-Irradiation and Subsequent Treatment with Topical All-Trans-Retinoic Acid on Skin Desmosine Content of Hairless Mice[a]

Weeks of Treatment	Control	Retinoic Acid
4	6.5 ± 1.5	5.3 ± 1.1
12	7.3 ± 0.6	6.6 ± 0.4
Non-irradiated	3.5 ± 0.6	
Irradiated	6.7 ± 0.4[b]	

[a] After irradiation animals (five per group) were treated topically three times per week with either acetone or 25 μg all-trans-retinoic acid. Units: ng desmosine/mm^2 skin. Values quoted are Mean ± SD (N = 5).

[b] $P < 0.01$ vs non-irradiated.

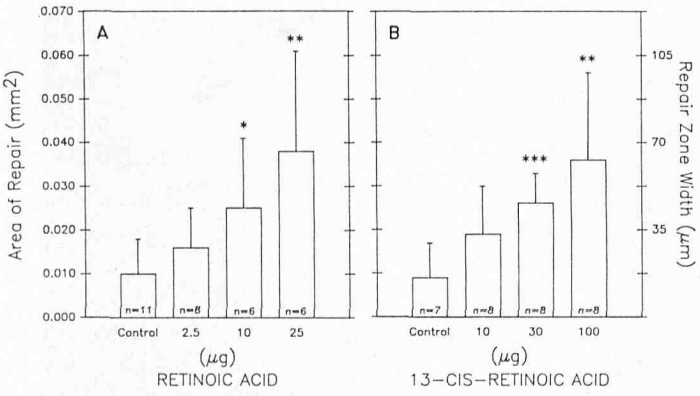

Figure 3. Repair of dermal damage in UVB-irradiated hairless mice by topically applied *(A)* all trans-retinoic acid and *(B)* 13-cis-retinoic acid. Animals were treated three times per week for 10 weeks. The numbers within the *bars* denote the group sizes. *P < 0.05; **P < 0.01; ***P < 0.001.

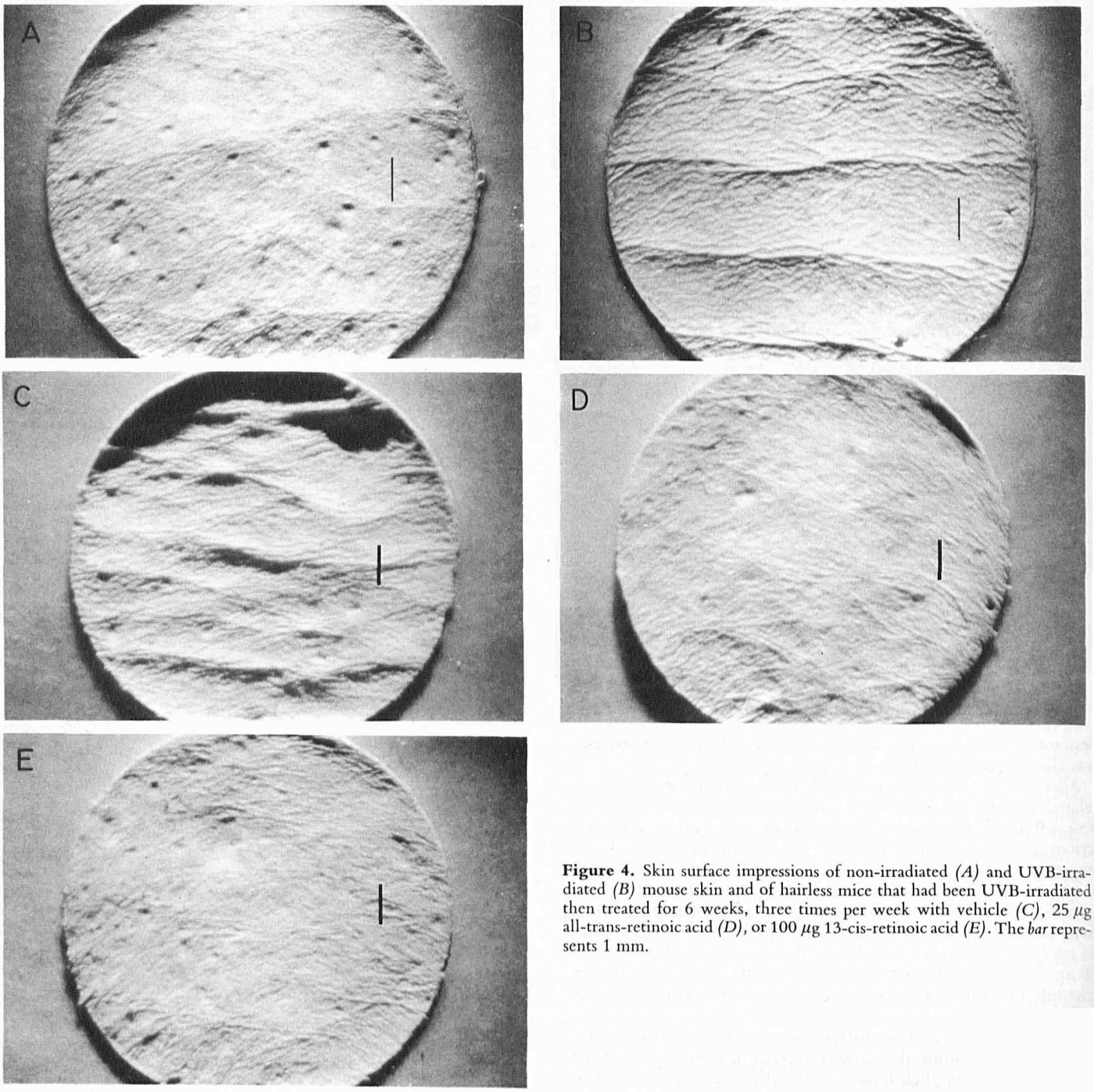

Figure 4. Skin surface impressions of non-irradiated *(A)* and UVB-irradiated *(B)* mouse skin and of hairless mice that had been UVB-irradiated then treated for 6 weeks, three times per week with vehicle *(C)*, 25 μg all-trans-retinoic acid *(D)*, or 100 μg 13-cis-retinoic acid *(E)*. The *bar* represents 1 mm.

retinoic acids for 6 weeks. The animals were about 6 months old and the total dose of radiation was 3.5 J/cm². Little regular structure is evident in non-irradiated animals (Fig 4*A*). After UVB irradiation, a new feature appears in the form of parallel lines spaced 2–3 mm apart (Fig 4*B*). The background is made up of an amorphous surface whose features may correspond to the pattern in the control animals but in a "swollen" state as a result of the epidermal hyperplasia caused by the radiation. These deep wrinkles do not appear to form at preferred locations determined by fine structure but rather they occur with the same spacing as the folds of excess skin in non-irradiated animals; indeed, in some cases (e.g., Fig 4*A*) the surface shows faint structures which would seem to be the sites of wrinkle formation. Chronic exposure of these sites of continual mechanical flexure to UVB somehow causes the formation of permanent creases that do not disappear upon gentle stretching.

After 6 weeks of treatment with topical retinoic acids, the line pattern is much less conspicuous and the skin reverts to a normal appearance. Figure 4*C* shows the skin surface patterns of vehicle control; Fig 4*D* animals were treated with 25 μg of all-trans-retinoic acid for 6 weeks; and Fig 4*E* animals were treated with 100 μg of 13-cis-retinoic acid for 6 weeks. Some effacement occurs with vehicle alone, but, as Fig 4*D, E* demonstrate, retinoic acid treatment brings about almost complete disappearance of the wrinkles. The remaining surface features resemble the background of untreated, irradiated samples, no doubt reflecting the influence of the thickened epidermis.

A dose-response effect can be demonstrated in the wrinkle effacement by quantitating the intensity of the line pattern. Figure 5 shows the dose-response data for the retinoic acids in which the doses were the same as those used to demonstrate repair of dermal damage. A monotonic response was observed with significant differences produced by 25 μg of all-trans-retinoic acid and by 30 and 100 μg doses of 13-cis-retinoic acid. As an index of relative potency, the doses that give 50% reduction in wrinkling can be estimated to be approximately 8 and 15 μg, respectively, which are in the range where effects on dermal damage were observed.

In some experiments surface impressions were recorded after 6, 8, or 10 weeks of treatment. At the lower dose of retinoid, i.e., 10 μg of 13-cis-retinoic acid, a definite time-dependence was observed in the extent of wrinkle effacement. Moreover, the rate of decrease in the Wrinkle Index was more than threefold greater for treated skin than for control.

When the animals were examined 5 weeks after retinoic acid treatment had been discontinued, the skin surface impressions showed that the wrinkles had remained effaced and no return to pre-treatment appearance was evident.

DISCUSSION

The age-related alterations in skin elastin have been well studied by light and transmission electron microscopy [5,12]. Scanning electron microscopy of aged skin [13] reveals a more dense network of elastic fibers in a more disorganized arrangement, particularly at the dermal/epidermal junction where the characteristic candelabra-like structures are absent [14,15]. It appears that a feature of cutaneous aging is an initial elastogenesis followed by a slow, spontaneous, progressive degeneration of elastic fibers leading, ultimately, to laxity and wrinkling. Mild actinic damage produces similar alterations in the elastin in humans [16] and the wrinkling accompanying photoaging strongly suggests a causal relationship between the integrity of the elastic fiber network and the mechanical properties of the skin.

Gross alteration in elastin clearly leads to the abnormal appearance of the skin in diseases such as anetoderma [17], Marfan syndrome [18], and elastoderma [19]; and a curious loss of elastic fibers from the mid-dermis, presumed to be the result of inflammatory elastolysis, led to widespread crinkling of the skin of a 42-year-old woman [20].

At a macroscopic level, wrinkles form when the underlying muscle contracts and the skin adapts itself by forming folds perpendicular to the line of contraction [21]. In humans, the habitual flexing of the facial skin leaves permanent furrows on the forehead and other areas around the eyes. The animal studied here, i.e., the hairless mouse, has excess dorsal skin which, with the animal at rest, lies in folds in the area above the tail; the spacing is determined by the thickness of the skin and the attachment to the underlying tissue. Upon gentle stretching, the folds are smoothed out leaving no residual line pattern. UVB-irradiated skin, on the other hand, retains a record of the folding even after stretching. In this regard they are wrinkles of the "permanent" type as defined by Tsuji et al [22].

There are no histologic correlates of such surface structures [20,21,23]; no structural features point to an area where mechanical flexure would be favored. Rather, constant flexing during continual UVB exposure has the effect of "fixing" the point of folding in the damaged, elastotic papillary dermis. A possible mechanism for this was suggested by Tsuji et al [22]: The valley of the wrinkle was partially shielded from UVB exposure by the surrounding thickened skin and thus incurred less damage. The authors claimed to observe less elastotic degeneration in the vicinity of a wrinkle in sun-exposed skin. However, sample preparation was by conventional formalin fixation and paraffin embedding, and it is difficult to understand how wrinkled skin could survive such harsh treatment and still show identifiable wrinkles.

The effects of retinoid treatment on wrinkling in the hairless mouse can be understood in the context of the repair of the dermal elastosis. The data presented here confirm the findings of a published study on retinoic acid [9] and extend the observations to include 13-cis-retinoic acid. To the extent that quantitative comparisons can be made, the results are similar. The values for the repair zone width reported here are less than published values; this discrepancy is almost certainly due to the inclusion, in our data analyses, of instances of zero repair, which would have the effect of reducing the final value. The two isomers of retinoic acid do not differ qualitatively in their effects on the histologic appearance of the tissue or on the wrinkling pattern produced. The all-trans isomer is slightly more potent in this system than the 13-cis isomer but substantially more irritating, which may limit the maximum degree of repair attainable. The "reconstructed" dermis is thickened, contains new collagen, and the tangled, disorganized elastin is packed into a thin layer in the lower dermis. Thus the framework within which a wrinkle had been established is eliminated and the skin assumes a normal state, as observed. That the effacement is apparently permanent is additional evidence of the relationship between the integrity of the elastic fiber network and the surface appearance. The only difference in the repaired skin (Fig 4*C–E*) is the absence of filamentous surface features. The thickening effect of UVB and subsequent retinoid treatment on the epidermis does not contribute substantially to the overall thickness of the repaired skin. Nevertheless, despite having a minor role in the effacement of deep wrinkles, these epidermal changes evidently preclude the formation of fine surface features.

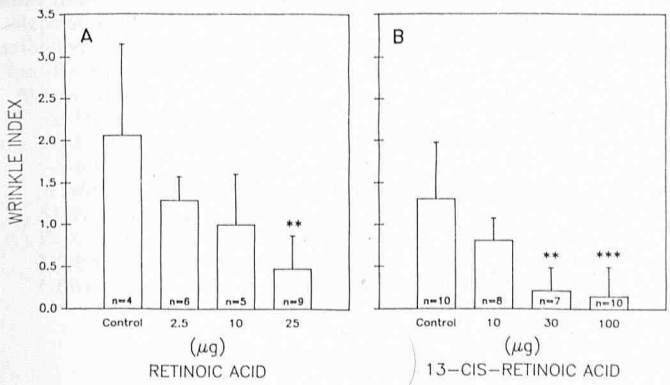

Figure 5. Dose-response data for wrinkle effacement by *(A)* all-trans-retinoic acid and *(B)* 13-cis-retinoic acid. Animals were treated as in Fig 3. The *numbers* within the *bars* denote the group sizes. $^{**}P < 0.01$, $^{***}P < 0.001$.

A recent study by Bissett et al [24] has shown similar effects of UV-B irradiation on surface features and histology in Skh-HR-I hairless mice. A zone of repair formed 16 weeks after radiation was stopped but no studies were reported on the effect of agents on acceleration of this process. It was concluded, however, that this system was a convenient model for testing the effectiveness of topical preventative treatments, such as sunscreens.

It is our opinion that this model is valid for the repair of photodamaged skin. From what is known about the role of elastin in maintaining skin integrity and from the association of wrinkling with excessive sun exposure, it is encouraging to observe the dual effect of retinoic acids. The smoothed appearance of the skin is not a transient, cosmetic adjustment but rather a return to a normal state as a result of fundamental biochemical changes occurring throughout the dermis.

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